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Comparative Transcriptional and Phenotypic Peripheral Blood Analysis of Kidney Recipients Under Cyclosporin A or Sirolimus Monotherapy

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Due to its low level of nephrotoxicity and capacity to harness tolerogenic pathways, sirolimus (SRL) has been proposed as an alternative to calcineurin inhibitors in transplantation. The exact mechanisms underlying its unique immunosuppressive profile in humans, however, are still not well understood. In the current study, we aimed to depict the *in vivo* effects of SRL in comparison with cyclosporin A (CSA) by employing gene expression profiling and multiparameter flow cytometry on blood cells collected from stable kidney recipients under immunosuppressant monotherapy. SRL recipients displayed an increased frequency of CD4 + CD25highFoxp3 + T cells. However, this was accompanied by an increased number of effector memory T cells and by enrichment in NFkB-related pro-inflammatory expression pathways and monocyte and NK cell lineage-specific transcripts. Furthermore, measurement of a transcriptional signature characteristic of operationally tolerant kidney recipients failed to detect differences between SRL and CSA-treated recipients. In conclusion, we show here that the blood transcriptional profile induced by SRL monotherapy *in vivo* does not resemble that of operationally tolerant recipients and is dominated by innate immune cells and NFkB-related pro-inflammatory events. These data provide novel insights on the complex effects of SLR on the immune system in clinical transplantation.

Key words: Calcineurin inhibitors, gene expression, immunosuppression, kidney transplantation, sirolimus, tolerance,

Abbreviations: PBMC, peripheral blood mononuclear cells; CSA, cyclosporin A; CNI, calcineurin inhibitor; SRL, sirolimus; mTOR, mammalian target of rapamycin; NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells; NK, natural killer; Th, helper T cell; Treg, regulatory T cell; IL, interleukin; STAT, signal transducer and activator of transcription; Tac, tacrolimus; PDN, prednisone; ALS, polyclonal anti-lymphocyte serum; OKT3, muronomab-CD3; CT, threshold cycle; FDR, false discovery rate; GVHD, graft-versus-host disease; PANTHER, Protein Analysis Through Evolutionary Relationships.

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Introduction

Following its introduction in the 1980s, cyclosporin A (CSA) had an enormous impact in organ transplantation by substantially decreasing acute graft rejection and improving long-term graft outcome (1). Central to these effects is the capacity of calcineurin inhibitors (CNIs) to interfere with T cell receptor signaling by blocking the translocation to the nucleus of nuclear factor of activated T cells and thereby preventing effective T cell activation, proliferation and survival (2). Administration of CNIs was, however, rapidly implicated in the development of severe metabolic and cardiovascular side effects such as diabetes, dyslipidemia, anemia and hypertension. Furthermore, CNIs provoke acute and chronic nephrotoxicity, which are risk factors for late kidney graft loss (3,4).

The discovery of inhibitors of the mammalian target of rapamycin (mTOR) such as sirolimus (SRL) provided a group

of new powerful immunosuppressants potentially capable of replacing CNIs and of exerting beneficial effects on chronic allograft nephropathy (5,6). Inhibition of mTOR neutralizes a series of cytokine-induced downstream signaling events and results in cell cycle blockade at the G1 to S transition and in the prevention of T cell proliferation (7). In addition, SRL also interferes with the expression of certain chemokine receptors on effector T cells and can directly affect other immune and nonimmune cell types (8–11). Thus, SRL but not CNIs, facilitates the selective expansion of CD4 + CD25highFoxp3+ regulatory T cells (Tregs) (12), can induce regulatory function in effector CD4+ T cells (13), and synergizes with co-stimulation blockade to induce allograft tolerance in stringent rodent models (13). Further, adoptively transferred SRL-conditioned monocyte-derived dendritic cells inhibit alloimmune responses and result in indefinite graft survival (14–16). Finally, mTOR inhibitors have intrinsic anti-tumor effects both in experimental animal models and in humans (17).

On the basis of these mechanisms of action, SRL has been utilized as a replacement for CNI in clinical kidney transplantation with three main rationales: (i) to avoid CNI nephrotoxicity and thus decrease chronic allograft nephropathy; (ii) to provide an anti-proliferative effect to recipients with neoplasic disorders and (iii) to enhance graft-protecting immunoregulatory pathways by promoting activation-induced apoptotic cell death of effector T cells and expanding alloantigen-specific Tregs. However, the initial enthusiasm concerning SLR's lack of nephrotoxicity when employed as an alternative to CNI was tampered down by the results of large randomized multicenter studies such as the Rapamune US Study Group (18) and the Elite Symphony Study (19) reporting that kidney recipients treated with SRL had higher 1-year serum creatinine levels than those treated with CNI. An intrinsic nephrotoxic effect of SRL was then demonstrated through several studies both in humans and in animal models (20–22). In parallel, a series of clinically significant and not well understood side effects of SRL were described, such as impaired wound healing, myelotoxicity, hypersensitivity-like interstitial pneumonitis, dyslipemia or diabetes (23), altogether accounting for the need to discontinue SRL treatment in at least 20% of kidney recipients. Finally, despite the observations made in experimental systems (24) whether SRL is more permissive for transplantation tolerance in the clinic than CNIs has not been formally proven yet.

Thus, the exact mechanisms underlying both the immunosuppressive profile of SRL and its side effects are still not well elucidated and continue to evolve. For instance, it was recently demonstrated that in monocytes and other innate immune cells inhibition of mTOR by SLR promotes the production of pro-inflammatory cytokines via the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) and simultaneously blocks interleukin (IL)-10 via signal transducer and activator of transcription (STAT)3 resulting in a strong helper T cell (Th)1 and Th17 polarization (8). Similarly, in contrast to the well documented anti-proliferative and pro-apoptotic effects exerted on naïve effector T cells, SRL has recently been shown to also act as an immunostimulatory reagent enhancing the generation of memory CD8+ T cells (25).

Clarification of the biological effects of SLR in clinical transplantation is particularly challenging, since the need to administer several immunosuppressants simultaneously hampers the precise delineation of the molecular pathways associated with individual agents. We hypothesized that an unbiased high-throughput molecular characterization of peripheral blood samples collected from stable kidney recipients under immunosuppressant monotherapy would reveal the predominant *in vivo* effect of these two drugs in human transplantation. Overall, our goal was therefore to identify markers of prolonged SRL therapy in peripheral blood of kidney recipients and employ them to understand the biological *in vivo* effects of SLR in comparison with kidney recipients under CSA monotherapy and with immunosuppression-free operationally tolerant patients.

Material and Methods

Patients and immunosuppressive therapy

Participating kidney recipients (n = 37) were enrolled from the Renal Transplant Unit, Hospital Clinic, Barcelona, Spain. The Ethical Committee approved the study and all patients gave their informed consent. Two groups of recipients were included: patients under CSA monotherapy (n = 13) and patients under SRL monotherapy (n = 24). SRL monotherapy was either started *de novo* or by converting patients on CNI-based regimens who had developed CNI-related nephrotoxicity. All enrolled patients fulfilled the following criteria: (1) deceased donor renal transplant recipients with >1 year posttransplant follow-up; (2) no significant variations in both serum creatinine and proteinuria over the previous 12 months; (3) immunosuppressive monotherapy with either CSA or SLR for at least 6 months (at the time of analysis) and (4) no acute rejection episodes over the previous 12 months; (5) no active neoplasic or infectious diseases. No evidences of systemic inflammatory responses (e.g. fever, elevated C-reactive protein and erythrocyte sedimentation rate) were apparent in any recipient at the time of analysis. Tables 1 and 2 summarize the characteristics of enrolled recipients. SLR and CSA recipients differed in donor age and in the time elapsed since transplantation, which reflects the fact that our Unit initiated the use SRL much later than that of CSA. Blood samples from a cohort of seven operationally tolerant recipients and seven age-matched nontolerant kidney recipients were also employed. Tolerant recipients were patients with stable kidney graft function (serum creatinine levels <1.7 mg/dL and proteinuria <1000 mg/24h) in the absence of immunosuppression for at least 1 year (range: 2–13 years) (26). Nontolerant patients were kidney recipients under standard immunosuppression with deteriorating kidney graft function (serum creatinine >1.7 mg/dL and/or proteinuria >1000 mg/24h) and exhibiting transplant glomerulopathy according to the updated Banff classification criteria and/or an active humoral component as demonstrated by the presence of graft C4d deposits and circulating anti-donor antibodies. In addition to kidney recipients, samples from a group of nine age-matched healthy individuals were also employed.

Peripheral blood immunophenotyping

Flow cytometry immunophenotyping was performed on whole blood samples collected from SRL and CSA recipients employing monoclonal anti-

Table 1: Demographics of SRL and CSA recipients

Study group	Recipient age at inclusion (years)[1]	Recipient gender	Donor age[1,*]	Time posttransplant[1,*] (years)	Graft number	A + B + DR mismatches	Serum creatinine at inclusion[1]	Proteinuria at inclusion[1]	Drug trough levels[2]
SRL (n = 4)	61 (8.9)	13 Male 11 female	50.3 (11.7)	9.75 (2.6)	23 First 1 third	≤2: 17 ≥3: 7	1.43 (0.35) mg/dL	710 (769) mg/day	10 (1.7) ng/mL
CSA (n = 3)	61.1 (14)	9 Male 4 female	23.8 (12.4)	16.4 (3.3)	11 First 2 second	≤2: 5 ≥ 3: 6 NA: 2	1.4 (0.6) mg/dL	519 (452) mg/day	119 (36) ng/mL

* p-Value <0.05.
[1] Mean (SD).
[2] Mean (SD) of drug trough levels obtained during the 6 months prior to inclusion.

bodies specific for the following markers: CD3, CD4, CD8, CD25, CD28, CD56, CD19, CD45RA, CD62L, CCR7, γδ TCR, αβ TCR (from BD Biosciences, San Jose, CA, USA); Vδ1 TCR (ThermoScientific, Waltham, MA, USA); Vδ2 TCR (Immunotech, Marseille, France) and Foxp3 (eBioscience, San Diego, CA, USA). Data acquisition was performed using a BDFacs Canto-II flow cytometer (BD Biosciences) and data analysis was conducted using FlowJo Software (Tree Star, Inc., Ashland, OR, USA). Statistical analysis was conducted employing the nonparametric Mann–Whitney test.

RNA extraction and cDNA preparation

RNA was extracted from peripheral blood using the TRIzol method (Invitrogen, Cergy Pontoise, France). Genomic DNA was removed by DNase treatment (Roche, Indianapolis, IN, USA). RNA quality and quantity was determined using an Agilent 2100 BioAnalyzer (Palo Alto, CA, USA). RNA was reverse transcribed into cDNA using polydT oligonucleotide and Maloney leukemia virus reverse transcription (Invitrogen).

Microarray experiments

Microarray experiments were conducted on peripheral blood mononuclear cells (PBMCs) isolated employing a Ficoll–Hypaque layer (Sigma-Aldrich, St. Louis, MO, USA). Total RNA was extracted with TRIzol reagent and the derived cRNA samples were hybridized onto Affymetrix Human Genome U133 Plus 2.0 arrays containing 54 675 probes for 47 000 transcripts (Affymetrix Inc., Santa Clara, CA, USA). Microarray data from all samples hybridized were normalized using the GC content adjusted-robust multiarray algorithm and a conservative probe-filtering step excluding those probes not reaching a log2 expression value of five in at least one sample was then applied. This resulted in the selection of a total of 22 586 probes out of the original 54 675 set. Significant Analysis of Microarray (SAM) (27) was employed to identify genes differentially expressed between the SRL and CSA groups and between the SRL group and the group of healthy volunteers. Microarray have been deposited in NCBI's Gene Expression Omnibus (28) and are accessible through GEO Series accession number GSE22224.

Functional analysis of gene expression data

To assess the deregulation of sets of genes associated with specific functional pathways we employed the Gene Set Enrichment Analysis (GSEA) method (29,30) on the filtered 22 586-probe set ranked according to SAM. This computational method determines whether an *a priori* defined gene set shows statistically significant concordant differences between two biological states. The gene sets used in this paper were obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database (http://www.genome.jp/kegg/pathway.html) and the Protein Analysis Through Evolutionary Relationships (PANTHER) classification system (http://www.pantherdb.org/pathway/). Furthermore, we employed blood cell lineage-specific transcripts identified in the recently reported Hematology Expression Atlas (31) to generate additional gene sets containing transcripts considered unique to CD4+ T and CD8+ T lymphocytes, CD14+ monocytes, CD19+ B lymphocytes, CD56+ natural killer (NK) cells and CD66b+ granulocytes. In addition to GSEA, we also utilized the Ingenuity Pathway Analysis Toxicology (IPA-Tox™; http://www.ingenuity.com) analysis to further explore the pharmacological response of PBMCs to either CSA or SRL treatment. This application employs molecular toxicity pathways and associated gene sets to delineate transcriptional responses to xenobiotic insults.

Quantitative RT-PCR

The gene signature of 'operational tolerance' originally consisted of 49 genes identified using custom cDNA microarrays by comparing operationally tolerant kidney recipients, chronic rejectors and healthy individuals

Table 2: Immunosuppressive regimens prior to the adoption of monotherapy

Study group	Induction therapy	Maintenance immunosuppression[1]	Treatment assignment
CSA	–	SRL + CSA	*De novo*
CSA	–	Tac + CSA	*De novo*
CSA	–	CSA	*De novo*
CSA	–	CSA	*De novo*
CSA	–	CSA	*De novo*
CSA	ALS	CSA	*De novo*
CSA	–	CSA	Conversion from Tac
CSA	ALS	CSA	*De novo*
CSA	ALS	CSA	*De novo*
CSA	–	SRL + PDN + CSA	Conversion from Tac
CSA	–	CSA	*De novo*
CSA	–	CSA + PDN	*De novo*
CSA	–	SRL + PDN	Conversion from SRL
SRL	–	SRL + CSA + CSA	Conversion from CSA
SRL	–	SRL + PDN	*De novo*
SRL	–	SRL + PDN	*De novo*
SRL	–	SRL + CSA	*De novo*
SRL	–	SRL + CSA	Conversion from CSA
SRL	–	SRL + PDN	*De novo*
SRL	–	SRL + CSA	Conversion from CSA
SRL	–	SRL + PDN	Conversion from CSA
SRL	–	SRL + CSA + PDN	Conversion from CSA
SRL	–	SRL + PDN	Conversion from CSA
SRL	–	SRL + CSA + PDN	*De novo*
SRL	–	SRL + CSA	Conversion from CSA
SRL	–	SRL + CSA	Conversion from CSA
SRL	–	SRL + PDN	Conversion from CSA
SRL	–	SRL + PDN	Conversion from CSA
SRL	–	SRL + PDN	Conversion from CSA
SRL	–	SRL + PDN	Conversion from CSA
SRL	–	SRL + PDN	*De novo*
SRL	–	SRL + PDN	Conversion from CSA
SRL	–	SRL + CSA	Conversion from CSA
SRL	–	SRL + PDN	Conversion from CSA
SRL	–	SRL + PDN	*De novo*
SRL	BAS	SRL + CSA	Conversion from CSA
SLR	OKT3	SRL	*De novo*

Tac = tacrolimus; PDN = prednisone; CSA = cyclosporine A; SRL = sirolimus; ALS = polyclonal anti-lymphocyte serum; BAS = basiliximab; OKT3 = muronomab-CD3.
[1]Maintenance immunosuppressive regimen 1 year prior to initiation of CSA or SRL monotherapy.

(32). For the purpose of analyzing this signature by quantitative RT-PCR (qPCR) primer and probe sets were manually re-designed. This resulted in nine genes having to be excluded due to poor efficacy or impossibility to design adequate probes. As a result, optimization was successful for the following 40 genes: *AKR1C1*, *AKR1C2*, *AREG*, *AURKA*, *BTLA*, *BUB1B*, *C1S*, *CCL20*, *CDC2*, *CDH2*, *CHECK1*, *DEPDC1*, *ELF3*, *GAGE*, *HBB*, *IGFBP3*, *LTB4DH*, *MS4A1*, *MTHFD2*, *NCAPH*, *NR2F1*, *PARVG*, *PCP4*, *PLEKHC1*, *PLXNB1*, *PODXL*, *PPAP2C*, *RAB30*, *RASGRP1*, *RBM9*, *RGN*, *RHOH*, *SLC29A1*, *SP5*, *SPON1*, *SYNGR3*, *TACC2*, *TLE4*, *TMTC3 and ZWILCH*. The expression patterns of this group of 40 target genes and the housekeeping gene *HPRT1* were measured in duplicates employing the ABI 7900 Sequence Detector System (PE Applied Biosystems, Foster City, CA, USA). To quantify the levels of mRNA we normalized the expression of the target genes to *HPRT1* and employed the ΔΔthreshold cycle (CT) method of relative quantification.

Identification of patients displaying a tolerance-related transcriptional pattern among CSA and SRL patients

To tentatively classify recipients included in the CSA and SRL groups as either tolerant or nontolerant employing the expression data from the 40 genes assessed by qPCR we employed the nearest shrunken centroid classifier implemented in the Predictive Analysis of Microarray (PAM) package (33). To do so we defined a training set comprising data from the seven tolerant and seven nontolerant kidney recipients and a test set that included all samples from the CSA and SRL groups. To estimate the classification accuracy of the gene signature on the training set, a 10-fold cross-validation was conducted by selecting the threshold associated with the lowest error rate and filtering the noisiest genes. The same threshold was then used for class prediction of a test set that comprised the 37 CSA and SRL patients. This procedure was repeated several times to compute the overall error.

Results

Recipients treated with SRL monotherapy exhibit increased numbers of circulating CD4 + CD25highFoxp3+ T cells and CD4+ effector memory T cells

The analysis of leukocyte subpopulations revealed that patients receiving SRL showed a significantly decreased number of total lymphocytes ($p = 0.0178$) and basophiles ($p = 0.0146$; data not shown). Within the T cell population, patients under SRL showed statistically higher percentages of $\alpha\beta$ T cells (Figure 1A) and lower percentages of $\gamma\delta$ T cells (Figure 1B) than patients receiving CSA. Furthermore, among the two main subpopulations of $\gamma\delta$ T cells (namely Vδ1 and Vδ2), SRL recipients exhibited a significantly decreased percentage of Vδ2 T cells (Figure 1C), with no differences being observed in Vδ1 T cells or in the Vδ1/Vδ2 T cell ratio (data not shown). In addition, neither the frequencies nor the absolute numbers of B cells (CD19+), NK cells (CD3-CD56+), NKT cells (CD3+CD56+), CD4+, CD8+ and $\alpha\beta$ + CD4− CD8− (DN) T cells significantly differed between the two groups of recipients (data not shown). In contrast, within the CD4+ T cell population SRL patients harbored significantly higher percentages of effector memory CD4+CD45RA−CCR7− T cells (Figure 2A) and lower percentages of naïve CD4+ CD45RA+ CCR7+ (Figure 2B) and central memory CD4+ CD45RA− CCR7+ (Figure 2C) T cells in comparison to patients under CSA monotherapy. In addition, SRL recipients displayed an increased percentage of CD4 + CD25highCD62L+ (Figure 2D) and CD4 + CD25highFoxp3+ T cells (Figure 2E). No statistical differences were detected between the two groups of recipients regarding CD8+ T cell subsets (data not shown).

Blood whole-genome expression profile in SRL recipients is dominated by genes involved in pro-inflammatory pathways and specific for innate immune cells

To assess the impact of the two different immunosuppressive regimens on peripheral blood whole-genome expression patterns we conducted a comparative analysis of the Affymetrix microarray data employing SAM. Using a false discovery rate (FDR) <5%, SAM yielded a total of 468 up-regulated and 586 down-regulated genes in the SRL as compared with the CSA group (Figure 3 displays the 50 most significant differentially expressed genes).

Functional pathway analysis using GSEA revealed that, together with genes involved in mTOR signaling, multiple functional clusters associated with pro-inflammatory pathways were significantly enriched in the SRL group (angiotensin-II signaling, cytokine and chemokine signaling, MAPK signaling pathway, TLR signaling pathway, RAS pathway, etc.). (Table 3 displays the gene sets significantly enriched in the SRL group at a nominal p-value <0.01 and FDR < 25%; no pathways were enriched in the CSA with this significance value). To determine which of these functional changes could be attributed to SRL monotherapy we compared SLR recipients with healthy individuals. Several of the previously identified pro-inflammatory pathways were also up-regulated in SRL recipients when compared to healthy individuals (e.g. TLR signaling pathway, adipocytokine signaling pathway, MAPK signaling pathway). Additional pathways found to be significantly associated with SLR in this analysis were ubiquitin proteasome, TGF-β, PI3K and apoptosis signaling (Tables 3 and 4 display the gene sets with significant enrichment scores at nominal p-value <0.01 and FDR <25%; no gene sets were positively associated with the healthy individual group or with the CSA group at this significance value). To further understand the biological responses of PBMCs to either CSA or SLR, we re-analyzed the differentially expressed genes employing the toxicity list capability of Ingenuity Pathway Analysis. Among previously defined pharmacological responses, this application identified NFkB signaling pathway as the top 'toxicological' response contained within the expression dataset ($p = 0.000943$; Table 5). Finally, to investigate whether the over-representation of pro-inflammatory pathways in SRL recipients could be attributed to subclinical allograft rejection, we compared between CSA and SRL patients the expression of a group of genes previously proposed as blood transcriptional biomarkers of kidney allograft rejection: *PRF1*, *GZMB*, *FASLG*, *CD40LG*, *IFNG*, *IL4*, *IL5*, *IL6*, *PSMB10*, *TLR4*, *MYD88* and *TRIB1* (34–40). None of these genes, however, were found to be differentially expressed between SRL and CSA recipients.

Different PBMC subsets contribute to SRL and CSA gene expression profiles

To investigate the contribution of different PBMC subsets to the differential gene expression profile between SRL and CSA blood samples, we performed GSEA analysis employing the Hematology Expression Atlas collection of cell lineage-specific gene sets. This analysis revealed that transcripts specific for either CD14+ or CD56+ cells were significantly over-represented in the SRL group ($p < 0.001$, FDR < 0.1% and $p < 0.03$, FDR < 2%, respectively). In contrast, the only cell lineage-specific transcripts significantly up-regulated in the CSA group were those associated with CD19+ and CD4+ T cells ($p < 0.001$ and FDR < 0.1% for both subsets).

Only a minority of recipients under SLR and CSA monotherapy display a signature of operational tolerance in peripheral blood

We measured by qPCR the expression of the 40 target genes related to operational tolerance in blood samples from the SRL and CSA recipients and employed the results to tentatively classify these recipients into tolerant and nontolerant categories. The optimal classifier obtained employing PAM included a total of 26 out of the 40 genes. This set of genes showed excellent performance in the

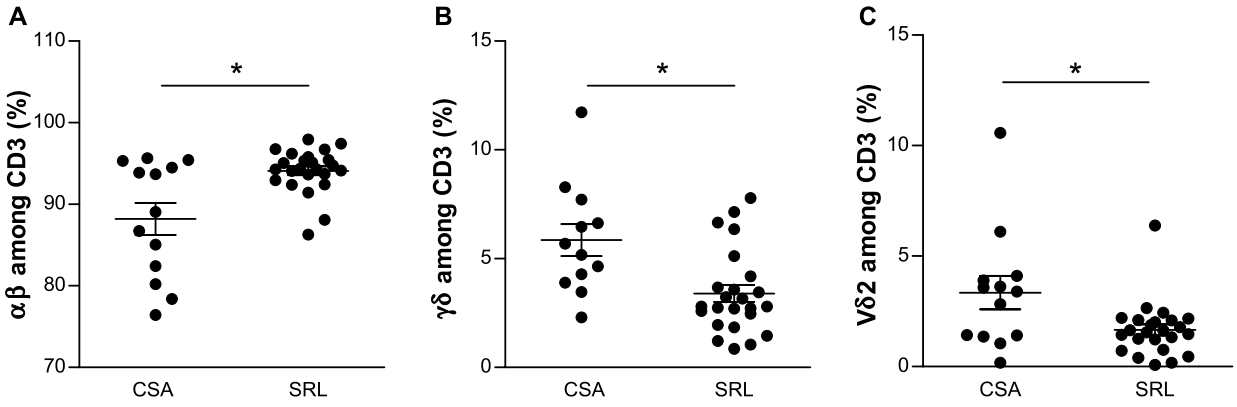

Figure 1: Differences in T cell populations between CSA and SRL recipients. Proportion of αβ TCR (A), γδ TCR (B) and Vδ2 (C) T cells among peripheral blood CD3+ mononuclear cells. Scattered dot plots represent mean (±SEM) values from 13 CSA and 24 SRL stable kidney recipients. (*), p-value < 0.05.

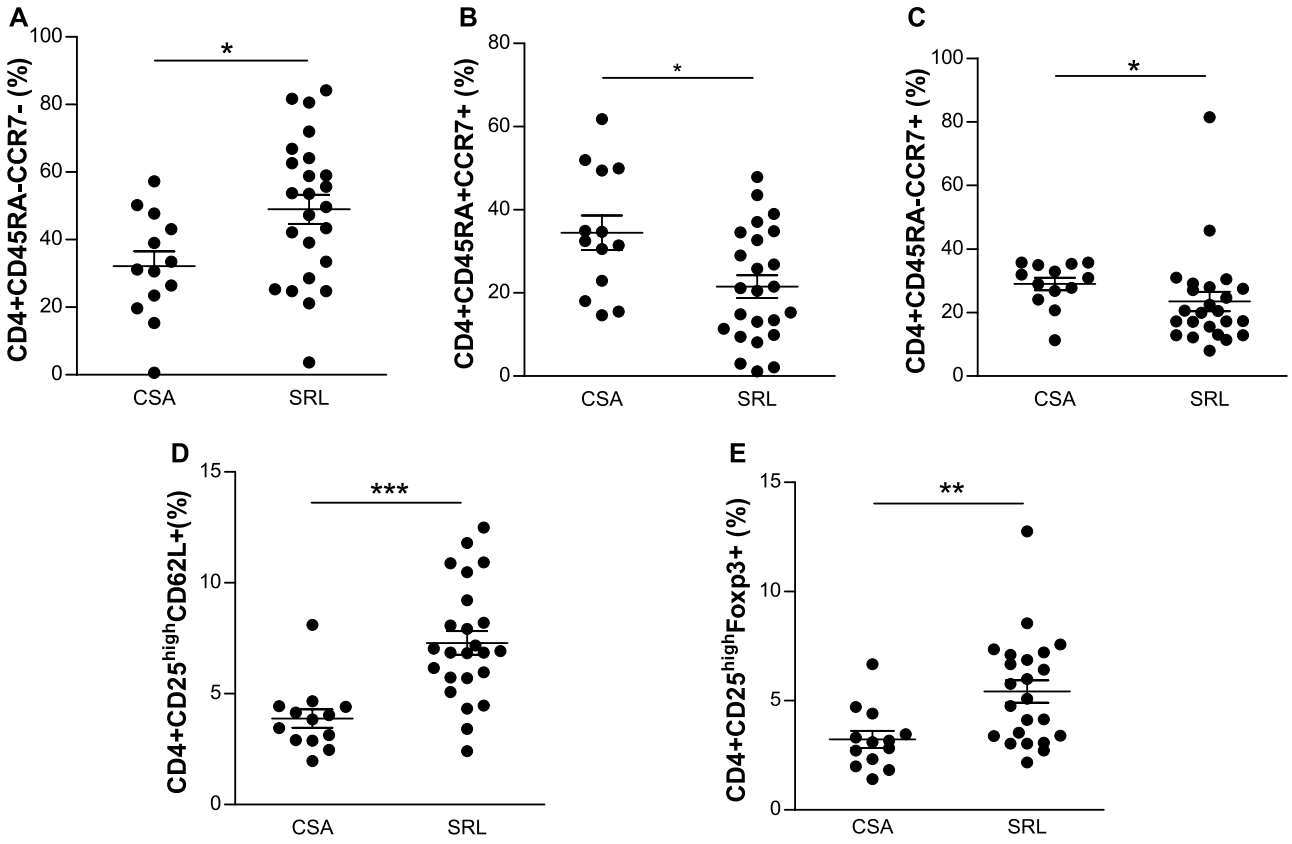

Figure 2: CSA and SRL recipients differ in CD4+ memory/naïve T populations and regulatory T cells. Comparison of the relative number of effector memory (T_{EM}) (A), naïve (B) and central memory (T_{CM}) (C) CD4+ T cells. Peripheral blood differences of CD4 + CD25highCD62L+ (D) and CD4 + CD25highFoxp3+ (E) T cells detected between CSA and SRL-treated cohorts. Scattered dot plots represent mean (±SEM) values. (*), p-value < 0.05; (**), p-value < 0.01; (***), p-value < 0.001.

training set by correctly classifying seven of seven tolerant samples and seven of seven nontolerant samples (overall success rate of 93.7%; data not shown). The use of this same classifier on the testing set resulted in four out of the 37 samples being predicted as potentially operationally tolerant: three patients under CSA monotherapy and one patient under SRL (data not shown). The four patients classified as potentially tolerant could not be

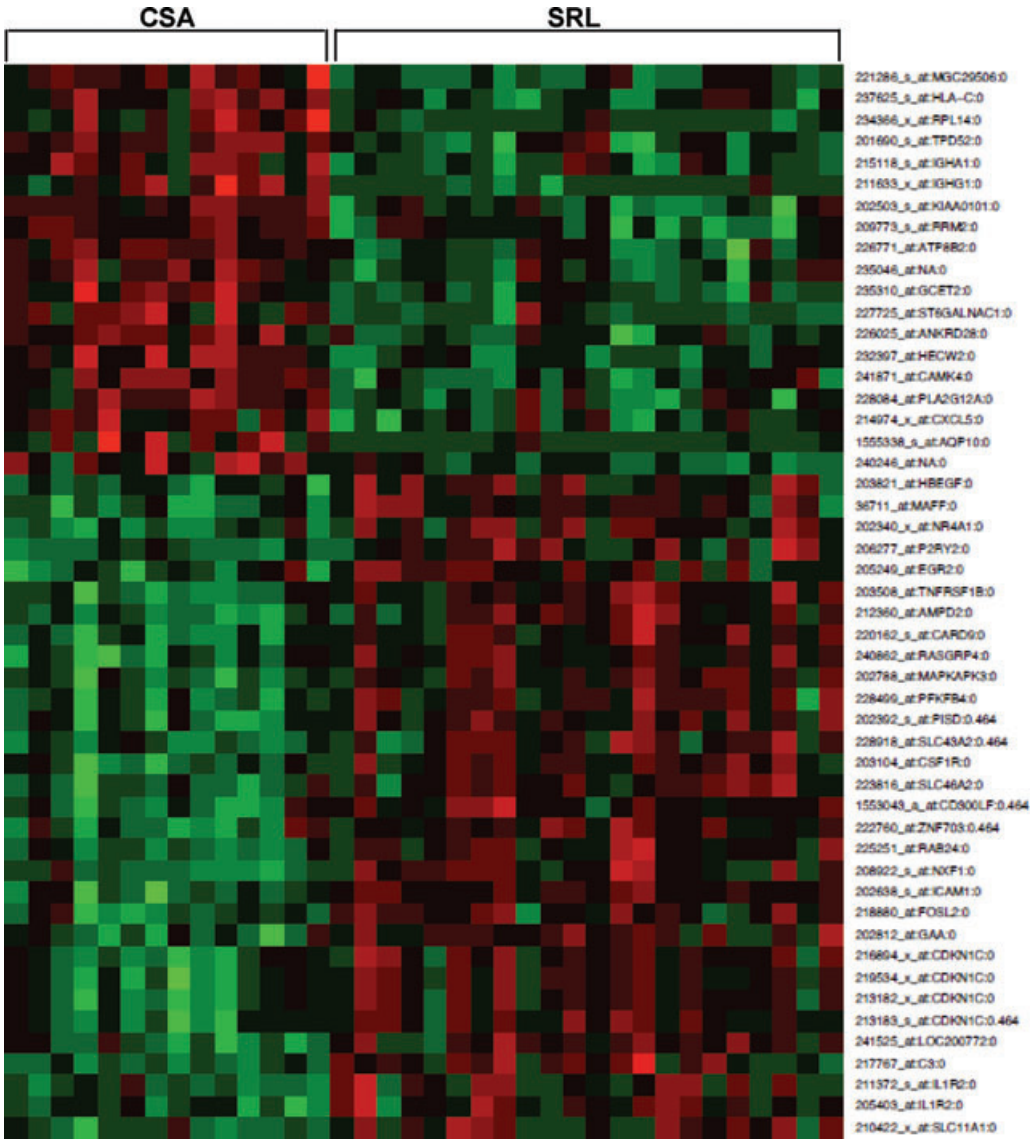

Figure 3: Whole genome expression profiling of PBMC samples reveals transcriptional differences between SRL and CSA recipients. Expression profile of the 50 most significant genes among the 1054 genes identified by SAM with FDR <5%. Results are expressed as a matrix view of gene expression (heatmap) where rows represent genes and columns represent samples. The intensity of each color denotes the standardized ratio between each value and the average expression of each gene across all samples. Red colored pixels correspond to an increased abundance of the transcript in the indicated sample, whereas green pixels indicate decreased transcript levels.

discriminated from the remaining 33 recipients on the basis of clinical or immunophenotypic characteristics.

Discussion

In this study, we have compared PBMC gene expression and phenotypic patterns from kidney recipients exhibiting stable graft function under SRL and CSA maintenance monotherapy to investigate the differential effects of these two immunosuppressive drugs *in vivo*. Patients receiving SRL displayed a significantly increased percentage of potentially regulatory CD4 + CD25highFoxp3+ T cells. However, the expansion of Tregs was accompanied by a significant increase in CD4+CD45RA–CCR7− effector memory T cells, which are lymphocytes particularly efficient at mediating allograft rejection (41). Several studies have reported that SRL, but not CNIs, contributes to the generation of high numbers of CD4+Foxp3+ Tregs *in vitro* and that these Tregs can be expanded without losing their suppressive activity and Foxp3 levels (42). Furthermore, studies in clinical renal transplantation have observed that SRL

Table 3: Functional pathways enriched in SRL as compared with CSA

KEGG pathways	Nominal p-Value	FDR q-Value	Representative genes with highest enrichment scores
Toll-like receptor signaling pathway	0.000	0.026	*TLR5*, *CD14*, *IL1B*, *CD86*, *NFKB2*, *TLR4*, *TLR2*, *IL8*
MAPK signaling pathway	0.000	0.029	*NR4A1*, *RASGRP4*, *IL1R2*, *MAPKAPK3*, *CD14*, *MPK7*, *TNFRSF1A*
Ephithelial cell signaling in *H. pylori* infection	0.000	0.053	*HBEGF*, *LYN*, *TCIRG1*, *ATP6V0C*, *NFKB2*
mTOR signaling pathway	0.001	0.042	*TSC2*, *STK11*, *LYK5*, *AKT1*, *EIF4BP1*, *ULK1*
Acute myeloid leukemia	0.003	0.042	*TCF7L2*, *RARA*, *SPI1*, *NFKB2*, *CEBPA*
Adipocytokine signaling pathway	0.005	0.054	*TNFRSF1B*, *RXRA*, *NFKB2*, *TNFRSF1A*, *NFKBIE*
Snare interactions in vesicular transport	0.004	0.052	*STX11*, *STX5*, *STX6*, *STX3*, *STX10*
Notch signaling pathway	0.008	0.076	*NOTCH1*, *PSEN1*, *CTBP2*, *NOTCH2*, *NCOR2*
Insulin signaling pathway	0.000	0.073	*PKM2*, *RAF1*, *ARAF*, *ACACA*, *TSC2*, *SOCS3*, *AKT1*
GNRH signaling pathway	0.002	0.081	*HBEGF*, *PLCB1*, *MAPK7*, *PTK2B*, *PLCB2*, *PRKCD*
Panther pathways	**Nominal p-Value**	**FDR q-Value**	**Representative genes with highest enrichment scores**
Angiotensin II-stimulated signaling through G proteins and b-arrestin	0.000	0.021	*ARRB2*, *RHOC*, *RHOB*, *PLCB1*, *PLCB2*
Inflammation mediated by chemokine and cytokine signaling pathway	0.000	0.018	*RELB*, *PTGS2*, *STAT3*, *RHOB*, *IFNGR2*, *IL8*, *IFNAR1*, *SOCS3*
Parkinson disease	0.000	0.021	*MAPK7*, *FGR*, *LYN*, *HCK*, *ADRBK2*
MAPK pathway	0.000	0.027	*MAPKAPK3*, *MAPKAPK2*, *EIF4E2*, *MAP3K5*, *IL1R1*, *MAP3K11*
RAS pathway	0.000	0.026	*MAPKAPK3*, *RHOC*, *RHOB*, *MAPKAPK2*, *PAK1*, *RAF1*, *RRAS*
P53 glucose deprivation pathway	0.009	0.046	*TP53*, *TSC2*, *AKT1*, *STK11*, *IGBP1*, *FRAP1*
Angiogenesis	0.000	0.052	*MAPKAPK3*, *TCF7L2*, *RHOC*, *RHOB*, *NOTCH1*, *PAK1*
Toll-like receptor signaling pathway	0.005	0.059	*PTGS2*, *TLR1*, *CD14*, *NFKB2*, *TLR4*, *RELB*, *RELA*, *NFKBIE*
Endothelin signaling pathway	0.001	0.061	*PTGS2*, *ARAF*, *MAPK7*, *ADCY7*, *PRKAR2A*, *MAP2K2*
WNT signaling pathway	0.000	0.058	*ARRB2*, *TCF7L2*, *TLE3*, *PLCB1*, *PLCB2*

preserves the frequency of circulating Treg cells, whereas CNIs decrease their numbers (12,43). SLR can also alter the polarization signals required for effector T cell differentiation. For instance, SLR has been reported to promote Th2-type polarization *in vitro* and both Th2 and Tc2 graft-protecting lymphocytes in an *in vivo* graft versus host disease (GVHD) model (44). Other studies have documented a critical role of mTOR in the generation of memory CD8+ T cells (25) and in the regulation of dendritic cell maturation (8,11,45). Our observations that SRL-treated recipients have a larger population of Tregs and memory CD4+ T cells than those under CSA are therefore consistent with previous reports on the pleiotropic effects of mTOR inhibition on T cell plasticity in response to environmental cues.

Our study also reveals that the gene expression profile differing between SRL and CSA or between SRL and healthy individuals is characterized by an over-representation of genes involved in pro-inflammatory pathways. To investigate if these transcriptional differences could be attributed to a previously defined pharmacological response we employed the Ingenuity Pathway Analysis Toxicology application that identified NFkB signaling as the pathway most significantly associated with the SRL-related dataset. The transcriptome of SRL recipients was also characterized by upregulation of monocyte and NK cell lineage-specific transcripts, in contrast to CSA recipients who displayed increased levels of B and CD4+ T cell-specific transcripts. Interestingly, this signature was observed despite no increase of the number of these cells *in vivo*, suggesting a 'real' footprint of these cell populations on the SRL-associated expression dataset. These transcriptional results, obtained employing standard unbiased functional enrichment strategies, are consistent with recent findings on the NFkB-mediated pro-inflammatory effects of SRL on innate immune cells *in vitro*. According to these reports (8,46,47), in freshly isolated human monocytes and myeloid dendritic cells stimulated through their Toll-like receptors, inhibition of mTOR results in increased production of IL-12, IL-6, IL-23 and TNF-α and decreased production of IL-10. These effects are mediated by an

Table 4: Functional pathways enriched in SRL as compared with healthy individuals

KEGG pathways	Nominal p-Value	FDR q-Value	Representative genes with highest enrichment scores
Porphirin and chlorophyll metabolism	0.001	0.028	*BLVRA, EPRS,FTH1, ALAS2, COX10*
Ubiquitin-mediated proteolysis	0.000	0.035	*UBE2E2, UBE2D2, CUL1, UBE2E1, UBE2D1*
Oxidative phosphorylation	0.000	0.049	*UQCRB, SDHC, ATP6V0C, COX10, NDUFV3*
O-glycan biosynthesis	0.001	0.040	*GALNT1, B4GALT5, C1GALT1, GCNT3, GALNT10*
Axon guidance	0.000	0.035	*PP3R1, DPYSL2, GNAI3, PAK1, KRAS, CDC42*
Neurodegenarative diseases	0.001	0.035	*PINK1, BCL2L1, SNCA, HSPA5, APP, NR4A2*
Adipocytokine signaling pathway	0.001	0.044	*STAT3, SOCS3, NFKBIA, CPT2, ACSL3*
Tight junction	0.002	0.077	*CSDA, GNAI3, PTEN, RAB13, EPB41L3, RRAS*
Toll-like receptor signaling pathway	0.000	0.069	*TLR5, IL1B, IFNAR1, NFKBIA, TLR4, PIK3CG*
Ephithelial cell signaling in *H. pylori* infection	0.004	0.070	*PAK1, ATP6V0C, LYN, HBEGF, CDC42*
WNT signaling pathway	0.003	0.071	*TCF7L2, PPP3R1, SMAD2, VANGL1, CUL1*
MAPK signaling pathway	0.000	0.065	*PPP3R1, STK3, DUSP3, ARRB1, DUSP1*
Apoptosis	0.002	0.070	*PPP3R1, CYCS, IL1B, BCL2L1, PRKACA*
Alzheimer disease	0.010	0.066	*APH1A, IL1B, SNCA, APP, GSK3B, PSEN1*
Pancreatic cancer	0.004	0.064	*STAT3, RALA, RALBP1, SMAD2, BCL2L1, KRAS*
ERBB signaling pathway	0.007	0.068	*CDKN1A, PAK1, HBEGF, KRAS, PRKCB1, PIK3CG*
Regulation of actin cytoskeleton	0.003	0.100	*PAK1, WASL, RRAS, PPP1R12A, MRLC2*
Panther pathways	**Nominal p-Value**	**FDR q-Value**	**Representative genes with highest enrichment scores**
Ubiquitin proteasome pathway	0.000	0.001	*PSMD1, UBE2E2, UBE2D2, UBE2D, PSMC2*
TGF-β signaling pathway	0.000	0.002	*RIT1, SMAD1, FOXK2, SMAD2, JUNB,SMAD3, RAB10*
Parkinson disease	0.000	0.002	*PSMA2, CUL1, LYM, PSMA4, MPAK7, ADRBK2, SNCA*
PI3 Kinase pathway	0.000	0.023	*FOXK2, GNAI3, RRAS, FOXJ2, PDPK1, KRAS, PIK3CG*
Apoptosis signaling pathway	0.000	0.056	*BCL2A1, ATF6, CYCS, ATF3, BCL2L1, NFKBIA, TNFSF10, REL*
Axon guidance mediated by semaphorins	0.006	0.054	*DPYSL2, PAK1, AKAP13, PAK2, PAF4, CDK5*

increased activity of the transcription factor NFkB and decreased activity of Stat3 (8) and result in myeloid antigen presenting cells being more effective at priming Th1 and Th17 adaptive immune responses (8). Our results reveal for the first time that when SRL is administered in monotherapy to stable kidney recipients, the overall effect on PBMCs is dominated by a pro-inflammatory bias even if recipients have no clinical evidences of systemic inflammation.

mTOR signaling was also identified among the functional pathways over-represented in SLR recipients. The up-regulated genes contained in this pathway included both effectors (*AKT1*) and inhibitors (*TSC2*, *EIF4EBP*) of mTOR signaling, which does not fit current models of mTOR signaling transduction. Under chronic SRL treatment compensatory mechanisms might try to restore the inhibited mTOR pathway with corresponding down-regulation of inhibitors and up-regulation of effectors. It is difficult, however, to estimate the downstream net effect on a complex signal-transduction pathway employing transcriptional data only. Our data clearly warrant a more detailed study on sorted PBMC populations with direct measurement of the phosphorylation status of key mediators of mTOR signaling.

mTOR inhibitors are universally considered as being more permissive to transplantation tolerance than CNIs. This is mostly based on data generated in experimental animal models in which, in contrast to CSA, SLR promotes allograft tolerance or at least does not hamper the induction of tolerance when administered in combination with tolerogenic reagents (15). In these models, tolerance is dependent on the graft-protecting effects of Tregs, which are inhibited in the presence of a pro-inflammatory milieu within the graft and draining lymph nodes (48–50). Having determined in our study that in kidney recipients the use of SRL monotherapy is associated with both expansion of Tregs and up-regulation of pro-inflammatory genes, we sought to investigate the overall effect of this agent in comparison to CSA on tolerance-associated markers. Thus, we analyzed

Table 5: Top toxicological responses identified through IPA-Tox

Name	p-Value
NFkB signaling pathway	0.000943
Mechanism of gene regulation by peroxisome proliferators via PPARα	0.001160
Hepatic cholestasis	0.011
LXR/RXR activation	0.011
VDR/RXR activation	0.0196

in PBMC samples from SRL and CSA recipients the expression of a set of transcriptional biomarkers previously shown to be differentially expressed in operationally tolerant kidney recipients as compared with both healthy individuals and recipients with immunologically driven chronic rejection (32). The same set of biomarkers was measured in blood samples collected from a cohort of seven operationally tolerant recipients. Importantly, these tolerant recipients were different from the original cohort employed to identify the transcriptional signature (32). The use of this signature correctly classified all seven tolerant recipients but failed to identify significant differences in the expression of tolerance-related genes between recipients treated with SRL and CSA monotherapy. This observation has to be taken with some caution, however, given that none of the biomarkers of kidney allograft tolerance reported in the literature (32,51,52) has demonstrated yet the capacity to identify tolerant individuals among immunosuppressed transplant recipients.

In conclusion, we show here that despite the well-documented anti-proliferative and pro-tolerogenic properties of SLR, the overall effects of this agent when administered in monotherapy to human kidney recipients are dominated by innate immune cells and NFkB-related pro-inflammatory events. While SLR treatment is associated with a larger pool of circulating potentially Tregs, it does not appear to confer a more 'tolerogenic' environment than that provided by CNIs. These data provide novel insight on the complex effects of SLR on the immune system of human transplant recipients. Although our results need to be confirmed in the context of prospective randomized studies, the conclusions of our study raise potential concerns about the use of SRL within tolerogenic regimens in clinical transplantation.

Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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